Determination of *Z*-Ligustilide and/or Ferulic Acid in Compound Chinese Medicine Granules by HPLC[1]

Safety precautions: *This procedure involves carcinogenic chemicals, corrosive chemicals and flammable solvents. Apply precautions when handling such chemicals, for example: use eye and hand protection and where necessary carry out the work in a fume cupboard to avoid inhalation of vapor.*

1. Introduction

1.1. Compound Chinese medicine granules are one of the proprietary Chinese medicines (pCm) sold in a variety of formulations in Hong Kong. Most of them are formulated in accordance with ancient Chinese medicine bibliographies. In general, they are produced by pulverising, decorating, filtering and concentrating a series of Chinese herbal medicines into a blend of water-soluble powders.

1.2. This method utilising the technique of high-performance liquid chromatography (HPLC) coupled with a diode array detector (DAD), is developed to analyse nine formulations with Radix Angelicae Sinensis (當歸) as the principal or assistant drug, or as a major component. The nine formulations are listed below.

Item	Formulation	Ancient Chinese Medicine Bibliography	Validated Analytes	
			Z-Ligustilide	Ferulic Acid
1.	聖愈湯	醫宗金鑒	✓	✓
2.	當歸四逆湯	傷寒論	✓	✓
3.	濟川煎	景嶽全書	---	✓
4.	補陽還五湯	醫林改錯	✓	✓
5.	蠲痹湯	濟生方	---	✓
6.	當歸芍藥散	金匱要略	✓	✓
7.	調經丸	證治準繩	✓	✓
8.	消風散	外科正宗	---	✓
9.	溫經湯	金匱要略	---	✓

1.3. This method has been validated for qualitative and/or quantitative determination of *Z*-ligustilide and ferulic acid in the nine formulations using HPLC-DAD. The validated working range for *Z*-ligustilide is 0.02–0.5 μg/mL in solution, which corresponds to 1–25 μg/g in the original sample, while that for ferulic acid is 0.02–0.5 μg/mL in solution, which corresponds to 50–1250 μg/g in the original sample.

2. Reagents

Note: *All reagents used should be of analytical reagent grade or equivalent unless otherwise specified.*

2.1. Water, Milli Q.

2.2. Acetonitrile (ACN), LC-MS grade.

2.3. Methanol (MeOH), LC-MS grade.

2.4. Phosphoric acid (H_3PO_4), ACS grade.

2.5. 0.1 % H_3PO_4 Solution

Mix ~1 mL of H_3PO_4 and ~999 mL of water in a 1-L measuring cylinder.

2.6. 50 % MeOH Solution

Mix ~500 mL of MeOH and ~500 mL of water in a 1-L measuring cylinder.

2.7. *Z*-Ligustilide, CAS. No.: 81944-09-4.

2.8. Ferulic acid, CAS. No.: 537-98-4.

2.9. Standard Solutions

2.9.1. Stock Standard Solution (~2000 µg/mL)

Weigh accurately ~20 mg of *Z*-ligustilide and ferulic acid into separate 10-mL volumetric flasks. Dissolve and make up to the graduation mark with MeOH.

2.9.2. Mixed Intermediate Standard Solution 1 (INT1, ~10 µg/mL)

Pipette 0.05 mL of the stock standard solutions into a 10-mL volumetric flask. Make up to the graduation mark with MeOH.

2.9.3. Mixed Intermediate Standard Solution 2 (INT2, ~1 µg/mL)

Pipette 2.5 mL of INT1 into a 25-mL volumetric flask. Make up to the graduation mark with MeOH.

2.10. Calibration Standard Solutions

Freshly prepare at least six calibration standard solutions from INT2. Make up with the mixture of 0.1 % H_3PO_4 solution and ACN (1:1, v/v) in 10-mL volumetric flasks. Suggested concentrations of calibration standard solutions used in the analysis are listed below.

Calibration Standard Solution	Vol. of INT2 (mL)	Final Vol. (mL)	Final Conc. of *Z*-Ligustilide and Ferulic Acid (μg/mL)
CS1	0.2	10.0	0.02
CS2	0.5	10.0	0.05
CS3	1.0	10.0	0.1
CS4	2.0	10.0	0.2
CS5	3.0	10.0	0.3
CS6	5.0	10.0	0.5

Remark: *Other concentrations may be used as appropriate. Other sequences of dilution may be employed to achieve the required concentrations. Record the details in the data sheets.*

2.11. Initial Calibration Verification (ICV) Standard Solutions

2.11.1. Stock ICV Standard Solution (~2000 μg/mL)

Prepare a stock ICV standard solution according to Clause 2.9.1 using an independent source of reference materials (another brand or another batch of standard with same brand as appropriate).

2.11.2. Mixed Intermediate ICV Standard Solution I (ICV I, ~10 μg/mL)

Pipette 0.05 mL of the stock ICV standard solutions into a 10-mL volumetric flask. Make up to the graduation mark with MeOH.

2.11.3. Mixed Intermediate ICV Standard Solution II (ICV II, ~1 μg/mL)

Pipette 1.0 mL of ICV I into a 10-mL volumetric flask. Make up to the graduation mark with MeOH.

2.11.4. ICV Working Solution (~0.1 or 0.2 μg/mL)

Pipette 1.0 or 2.0 mL of ICV II into a 10-mL volumetric flask. Make up to the graduation mark with the mixture of 0.1 % H_3PO_4 solution and ACN (1:1, v/v).

2.12. Calibration Check Standard

Calibration check standard shall be the CS3 or CS4 of the calibration standard solutions, *viz.* ~0.1 or 0.2 μg/mL.

2.13. Method Blank

The method blank shall be carried out through the complete sample preparation procedures as per Clauses 4.1.3 to 4.2 and shall contain the same amount of reagents as the sample solutions.

3. Apparatus

Note: *All glassware shall be rinsed with acetone and washed with detergent solution as soon as practicable after use. After detergent washing, glassware shall be rinsed immediately, firstly with water and then with acetone twice.*

3.1. Grinder or blender.

3.2. Volumetric flasks, 10- and 25-mL.

3.3. Measuring cylinder, 1-L.

3.4. Analytical balance, capable of weighing to 0.01 mg.

3.5. Autopipettes, 300-, 1000-µL and 10-mL.

3.6. Centrifuge tubes, 15-mL with polypropylene screw cap.

3.7. Ultrasonic bath.

3.8. Vortex mixer.

3.9. High speed centrifuge with rotation speed settable at least 4000 rpm.

3.10. PTFE membrane filters, 0.20-µm or equivalent.

3.11. LC glass vials.

3.12. HPLC column, Zorbax Eclipse Plus C_{18} Column (4.6 × 250 mm, 5 µm) or equivalent.

3.13. HPLC-DAD, Dionex UltiMate 3000 HPLC System or equivalent performance.

4. Procedures

4.1. Sample Preparation

4.1.1. Grind and homogenise solid samples using grinder or blender before analysis, if necessary.

4.1.2. Accurately weigh ~0.5 g of the sample into a 15-mL screw cap centrifuge tube.

4.1.3. Add 10 mL of 50 % MeOH solution into the centrifuge tube. Vortex the sample mixture in the centrifuge tube for ~1 min.

4.1.4. Sonicate the sample mixture in an ultrasonic bath for ~20 min at room temperature.

4.1.5. Centrifuge the sample mixture at ~4000 rpm for ~10 min to settle the residue. Transfer the supernatant solution to a 25-mL volumetric flask.

4.1.6. Repeat Clauses 4.1.3 to 4.1.5 **twice** with 4 mL of 50 % MeOH solution.

4.1.7. Collect all supernatant in the same 25-mL volumetric flask and make up to mark with 50 % MeOH solution.

4.1.8. Filter the sample solution with 0.20-μm PTFE filter disc. Collect the filtrate in a LC glass vial. The sample solution is ready for the analysis of *Z*-ligustilide.

4.1.9. For the analysis of ferulic acid, further dilute the sample solution 50 times with the mixture of 0.1 % H_3PO_4 solution and ACN (1:1, v/v).

Remark: *Further dilute the sample solution with the mixture of 0.1 % H_3PO_4 solution and ACN (1:1, v/v) if the concentration of analyte(s) is not within the calibration range.*

4.2. Analysis of HPLC-DAD

4.2.1. Operate the HPLC-DAD system in accordance with the instrument manual. Carry out analysis with conditions as suggested below. It may be necessary to modify the operational conditions for optimal signal output. Record the actual experimental conditions in the worksheet.

4.2.2. Suggested HPLC-DAD conditions:

HPLC System	**:**	Dionex UltiMate 3000 HPLC System or equivalent performance
Column	**:**	Zorbax Eclipse Plus C_{18} Column (4.6 × 250 mm, 5 μm) or equivalent
Mobile Phase A	**:**	0.1 % H_3PO_4 Solution
Mobile Phase B	**:**	ACN
Elution Program	**:**	Time (min) / A (%) / B (%) 0.0 / 97 / 3 18.0 / 97 / 3

		20.0	95	5
		25.0	95	5
		30.0	80	20
		35.0	60	40
		40.0	50	50
		45.0	50	50
		47.0	10	90
		60.0	5	95
		65.0	5	95
		66.0	97	3
		75.0	97	3
Flow Rate	**:**	0.5 mL/min		
Injection Volume	**:**	10 µL		
Reported Retention Time	**:**	~41.4 min for ferulic acid ~54.9 min for *Z*-ligustilide		
Column Temperature	**:**	25 °C		
DAD wavelength	**:**	320 nm		

5. Calculation/Result Interpretation

5.1. Identification Requirement

Identify the analyte(s) in the sample by comparison of the retention time of the detected peak (RT_{sample}) with that of the average retention time (RT) from calibration standards. The RT_{sample} shall not differ from that of the average RT from calibration standards by more than 5 % for positive identification.

5.2. Establish the calibration curve by plotting the peak area against the concentration of analyte(s) in linear calibration mode. Obtain the slope, y-intercept and the coefficient of determination (r^2) from the calibration curve.

5.3. Calculate the concentration of analyte(s) in sample (µg/g) as follows:

$$\text{Concentration of analyte(s) (µg/g)} = \frac{C \times V \times D}{W}$$

where C = Concentration of analyte(s) obtained from calibration curve (µg/mL);
V = Final volume (mL);
D = Dilution factor; and
W = Sample weight (g).

6. Reference

6.1. Chinese Pharmacopoeia Commission. (2025). *Pharmacopoeia of the People's Republic of China.* China Medical Science Press.

---

1 This method intends to provide a reliable analytical method that can be used as a quality control method for determining the chemical marker(s) in the corresponding pCm product(s). It is the user's responsibility to assess the suitability of testing their pCm product(s) when adopting this method.

– This is a blank page –